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Estrogen Promotes Mammary Tumor Development in C3(1)/SV40 Large T-Antigen Transgenic Mice: Paradoxical Loss of Estrogen Receptorα Expression during Tumor Progression

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ABSTRACT

Although several lines of epidemiological evidence suggest that estrogen exposure influences the incidence of breast cancer development, the mechanisms by which estrogen may stimulate the formation of breast cancer remain poorly understood. We have explored how alterations in estrogen exposure can influence the development of mammary cancer in the C3(1)/T_{AG} transgenic model, where estrogen levels and estrogen receptor α (ERα) expression do not appear to modify the level of transgene expression. The C3(1)/T_{AG} transgene becomes transcriptionally active in mammary ductal target cells at 3 weeks of age after the estrogen-induced differentiation of the mammary epithelial *anlage* to the ductal outgrowth stage. Complete maturation of the mammary ductal tree, however, is not required for cancer development because tumors arise in animals where ductal branching and terminal end bud formation have been prematurely arrested by ovariectomy. Mammary tumorigenesis in this model is promoted by increased estrogen exposure with the development of significantly more mammary intraepithelial neoplastic lesions and carcinomas associated with accelerated malignant conversion. The promotion of mammary tumors in this model appears to occur through an estrogen-induced proliferation and increase in the number of available target cells for transformation at the terminal ductal lobular units, as has been postulated to occur in women who receive hormone replacement therapy and/or by additional molecular mechanisms. We show, for the first time in a transgenic mouse model, that mammary tumor progression is associated with the loss of ERα expression, as has been often observed in human breast cancers with important clinical significance. Estrogen signaling may, therefore, serve different functions, depending upon the stage of tumorigenesis. ERβ expression is up-regulated during tumor progression, although the functional significance of this remains to be determined.

INTRODUCTION

The morbidity and mortality from breast cancer in women have been increasing in Western countries without a clear understanding of the etiological factors involved (1). Epidemiological studies have identified several risk factors for human breast cancer, such as early menarche, late menopause, and postmenopausal obesity, whereas protective factors include early full-term pregnancy, lactation, and physical exercise. A recent study has demonstrated that hormone replacement therapy increases the risk of developing breast cancer (2). Collectively, this epidemiological evidence suggests that the cumulative estrogen dose to the breast epithelium correlates with the risk of breast cancer (3).

ER[4] status is an important factor in assessing prognosis and in determining therapeutic strategies for treating breast cancer. Approximately 70% of human breast cancers stain positively for ERα. ERα expression is associated with more differentiated and less aggressive tumors. On the other hand, tumors lacking ERα are generally associated with a more aggressive disease course and poorer clinical outcome (4, 5). Tumors from the same patient often progress from an ERα-positive to an ERα-negative state. Therefore, breast cancers likely evolve into a hormone-unresponsive state and become poorly responsive to endocrine therapies.

To gain further insights into the role of estrogen signaling in mammary carcinogenesis, we have studied how alterations in estrogen exposure might influence the development of mammary cancer in the C3(1)/T_{AG} transgenic mouse model in which 100% of female mice develop mammary carcinomas, as reported previously (6). In this model, both large T- and small t-antigens of the SV40 early region are expressed under the transcriptional control of the 5′ flanking region of the C3(1) component of the rat prostate steroid binding protein gene. Although male mice carrying this transgene develop prostate cancer, the C3(1)/T_{AG} transgene is also expressed in both mammary epithelial ductal cells and the TLDUs without the need for pregnancy or hormone stimulation to drive transgene expression, as is often required for other transgenic mammary models (reviewed in Ref. 7). The mammary lesions arising in these transgenic mice develop over a predictable time course with histological similarities to human breast cancer (8, 9).

Unlike other transgenic models that use promoters that are highly responsive to hormone stimulation, we demonstrate that the C3(1) transgene is not estrogen responsive in the mammary gland. However, estrogen appears to promote mammary tumorigenesis in this model. The generation of C3(1)/T_{AG} mice lacking the ERα receptor demonstrates that mammary oncogenesis in this model requires the differentiation of the mammary ductal epithelium through the ductal outgrowth stage. Complete development and differentiation of the mammary ductal tree, however, are not required for tumorigenesis. In this study, we are the first to demonstrate, in a transgenic mouse model, that ERα expression appears to be lost during mammary tumor progression, as often occurs in human breast cancer. This transgenic model should provide further insights into the mechanisms of how ERα expression is lost during mammary cancer development and how estrogen may stimulate mammary oncogenesis.

MATERIALS AND METHODS

Cell Lines and Transient Transfection Assays. The mammary carcinoma cell line M6 was established from C3(1)/T_{AG} transgenic mammary carcino-

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[4] The abbreviations used are: ER, estrogen receptor; E_2, 17β-estradiol; MIN, mammary intraepithelial neoplastic; TAM, tamoxifen; OVX3W and OVX8W, ovariectomy at 3 and 8 weeks of age, respectively; TDLU, terminal ductal lobular unit; RT-PCR, reverse transcription-PCR; TGF, transforming growth factor; EGFR, epidermal growth factor receptor; MMTV, mouse mammary tumor virus; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

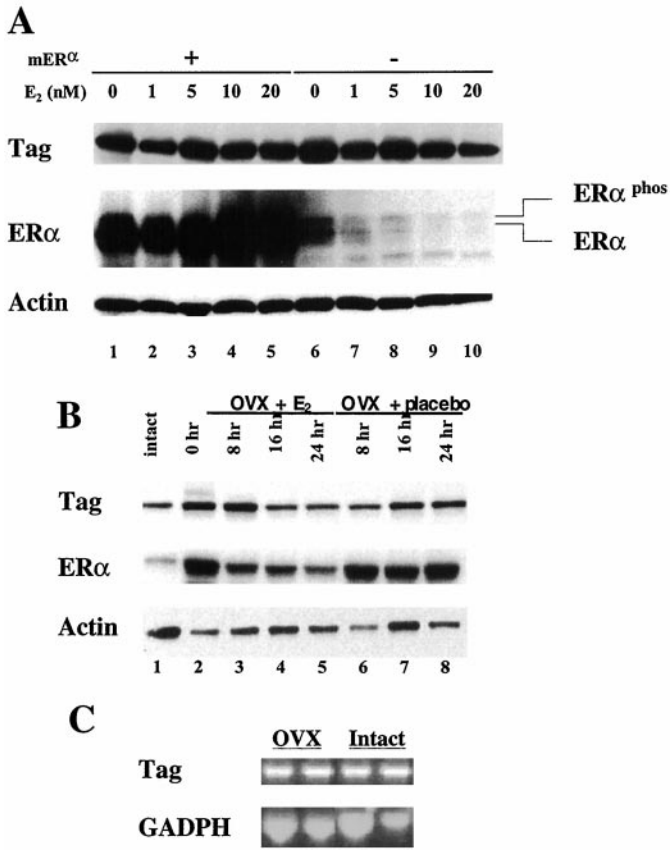

Fig. 1. Estrogen does not stimulate the C3(1)/T_{AG} transgene expression *in vitro* or *in vivo*. Western blot analysis is shown. *A*, T_{AG} levels do not change while ERα is down-regulated by estrogen in a dose-dependent manner in M6 cells. Cells were transfected with vector (−, *Lanes 1–5*) or with CMV-mERα [pSG5-MOR] (+, *Lanes 6–10*) and treated with 0–20 nM E_2. *ERα^{phos}* is the phosphorylated form of ERα. β-actin (*Actin*) is an internal control. *B*, T_{AG} expression is not altered by E_2 administration *in vivo*, although ERα is down-regulated by E_2, demonstrating a functional ER signaling pathway. Ten-week-old C3(1)/T_{AG} transgenic female mice, ovariectomized at 9 weeks of age, received vehicle alone (*Lanes 3–5*) or 3 mg/kg E_2 (*Lanes 6–8*) 1 week after ovariectomy. *Lane 1*, intact FVB/N mammary gland at 10 weeks of age. *C*, Tag expression in tumors that develop in OVX3W mice is similar to that for tumors from intact animals.

ma.[5] Cells were routinely maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum plus 5 ml of antibiotic-antimycotic solution (Life Technologies, Inc.) in an air:carbon dioxide (95:5) atmosphere at 37°C. E_2 was purchased from Sigma Chemical Co. (St. Louis, MO). For transfection assays, M6 cells were seeded in six-well plates in DMEM supplemented with 2% dextran-coated, charcoal-stripped fetal bovine serum (Life Technologies, Inc.) and grown until 70% confluent. The M6 cells were transfected with the mouse ERα plasmid (pSG5-MOR) using the FuGENE6 transfection reagent according to the manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN). The cells were treated with 0, 1, 2, 5, 10, or 20 nM of E_2 for 48 h.

Western Blotting. Cells were lysed with radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 mg/ml phenylmethylsulfonyl fluoride). Twenty μg of total protein were loaded onto 8% Tris-glycine gels (Novex, San Diego, CA) and transferred to nitrocellulose membranes (Novex, San Diego, CA). Membranes were incubated with 3% bovine serum albumin (Sigma), followed by the addition of primary antibody against SV40 T_{AG} (Ab-2, at a concentration of 1.0 μg/ml; Calbiochem, La Jolla, CA) or rabbit polyclonal anti-ERα antibody (MC20, at a dilution of 1:250; Santa Cruz Biotechnology, Santa Cruz, CA). Monoclonal anti-actin antibody (N350, at a dilution of 1:500; Amersham Life Science, Arlington Heights, IL) served as an internal control. Blots were then washed and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat antimouse or antirabbit IgG (CalT_{AG}; San Francisco, CA). Autoradiography was performed using the enhanced chemiluminescence method (NEN Life Science Products, Inc., Boston, MA).

Hormone Manipulations of C3(1)/T_{AG} Transgenic Mice. Transgenic mice carrying the C3(1)/T_{AG} transgene have been described previously by our laboratory and have been maintained in the FVB/NCr background (6). All studies were performed with female mice heterozygous for the transgene. The effects of altered estrogen signaling on tumor development were determined in response to various manipulations of the mice. For studies to determine the short-term effect of estrogen on transgene expression *in vivo*, mice were ovariectomized at 10 weeks of age. One week later, the mice received injections intraperitoneally with 3 mg/kg body weight of E_2 dissolved in 10% ethanol and 90% peanut oil (Sigma) or with peanut oil alone. Mice were sacrificed at 8, 16, or 24 h after injection, and mammary tissues were excised and frozen at −80°C for further analyses. Ten-week-old normal FVB/N female mice were implanted with pellets that release 0.23 mg of E_2 daily and sacrificed after 1 or 8 days for mammary gland analyses.

For studies to determine the long-term effects of hormone manipulations on tumorigenesis, groups of mice were ovariectomized prior to maturation but during a stage of ductal outgrowth at either 3 weeks of age (OVX3W), after puberty at 8 weeks of age (OVX8W), or left intact and implanted with placebo pellets (Innovative Research of America, Sarasota, FL). Additional groups of animals ovariectomized after sexual maturation at 8 weeks of age were either implanted with pellets (Innovative Research of America) containing 7.5 mg of E_2 released over 60 days (OVX E_2) or implanted with pellets containing 25 mg of TAM released over 60 days (Innovative Research of America). Pellets were replaced every 60 days. All manipulations of mice were performed in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, 1985, under animal protocol LC-059). Individual body weights were recorded weekly. Mammary tumor size was measured twice weekly using a caliper, and tumor volume was calculated using the following formula: the largest diameter × (the smallest diameter)^2 × 0.4 (10). Mice were euthanized by CO_2 asphyxiation.

Generation of C3(1)/T_{AG} Transgenic Mice Carrying Mutant ERα Alleles. To study the effect of ERα on mammary tumorigenesis in C3(1)/T_{AG} transgenic mice, C3(1)/$T_{AG}{}^{+/-}$ transgenic male mice in the FVB/N background were crossed with wild-type C57BL/6 female mice, and male offspring carrying the C3(1)/$T_{AG}{}^{+/-}$ transgene were identified by slot blot. These hybrid C3(1)/T_{AG} (FVB/N × C57BL/6) male mice were crossed with *ER$\alpha^{+/-}$*; C57BL/6 female mice. Males from the second cross with the C3(1)/$T_{AG}{}^{+/-}$; *ER$\alpha^{+/-}$* genotype were back crossed with *ER$\alpha^{+/-}$;C57BL/6 female mice. C3(1)/$T_{AG}{}^{+/-}$ female progeny with similar genetic backgrounds carrying either ER$\alpha^{+/+}$, ER$\alpha^{+/-}$*, or *ER$\alpha^{-/-}$* genotypes were used for analyses. Genotypes were identified from tail DNA by slot blot analysis using a probe for T_{AG} (6) and PCR to determine the ERα genotypes as described previously (11).

Whole-Mount Preparation, Histopathology, and Immunohistochemistry. Mammary gland whole-mount preparations were spread on a glass slide, fixed in 70% ethanol, rehydrated in distilled water, stained with 0.2% carmine and 0.5% aluminium potassium sulfate, redehydrated in 100% ethanol, cleared in xylene, and mounted with coverslips using Permount (Fisher Scientific, Fair Lawn, NJ). Mammary tissue and tumor samples were dissected from C3(1)/T_{AG} transgenic female or age-matched nontransgenic FVB/N females. The sizes of the lesions were recorded. A portion of each mammary tissue sample was also immediately frozen on dry ice for subsequent molecular biological analyses. The remaining mammary tissue was fixed in 4% paraformaldehyde or 10% phosphate-buffered formalin, embedded in paraffin, cut at a thickness of 4 μm, and stained with H&E for histopathological examination.

Histopathological lesions were quantitated by summing the number of lesions in sections from two axillary and one inguinal mammary gland from each mouse. Glands from the same locations in all mice were used for these studies.

For selected immunohistochemical analysis, the sections were heated by microwave in distilled water for antigen retrieval, hybridized with primary antibody, and processed using the avidin-biotin complex method (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA). Anti-SV40 T_{AG} mouse monoclonal antibody (PAB 101; PharMingen, San Diego, CA) was used at a dilution of 1:50. Anti-ERα rabbit polyclonal antibody (MC20; Santa Cruz Biotechnology) was used at a dilution of 1:500; and anti-proliferating cell nuclear antigen mouse monoclonal antibody (PC-10; Dako Corp., Carpinteria, CA) was used at a dilution of 1:500.

RNA Extraction, Northern Blotting, and Reverse RT-PCR Assay. RNA was extracted twice with RNA STAT-60 reagent (Tel-Test "B", Inc., Friendswood, TX) according to the manufacturer's protocol. RNA from mammary

[5] C. L. Jorcyk, M. Anver, and J. E. Green, manuscript in preparation.

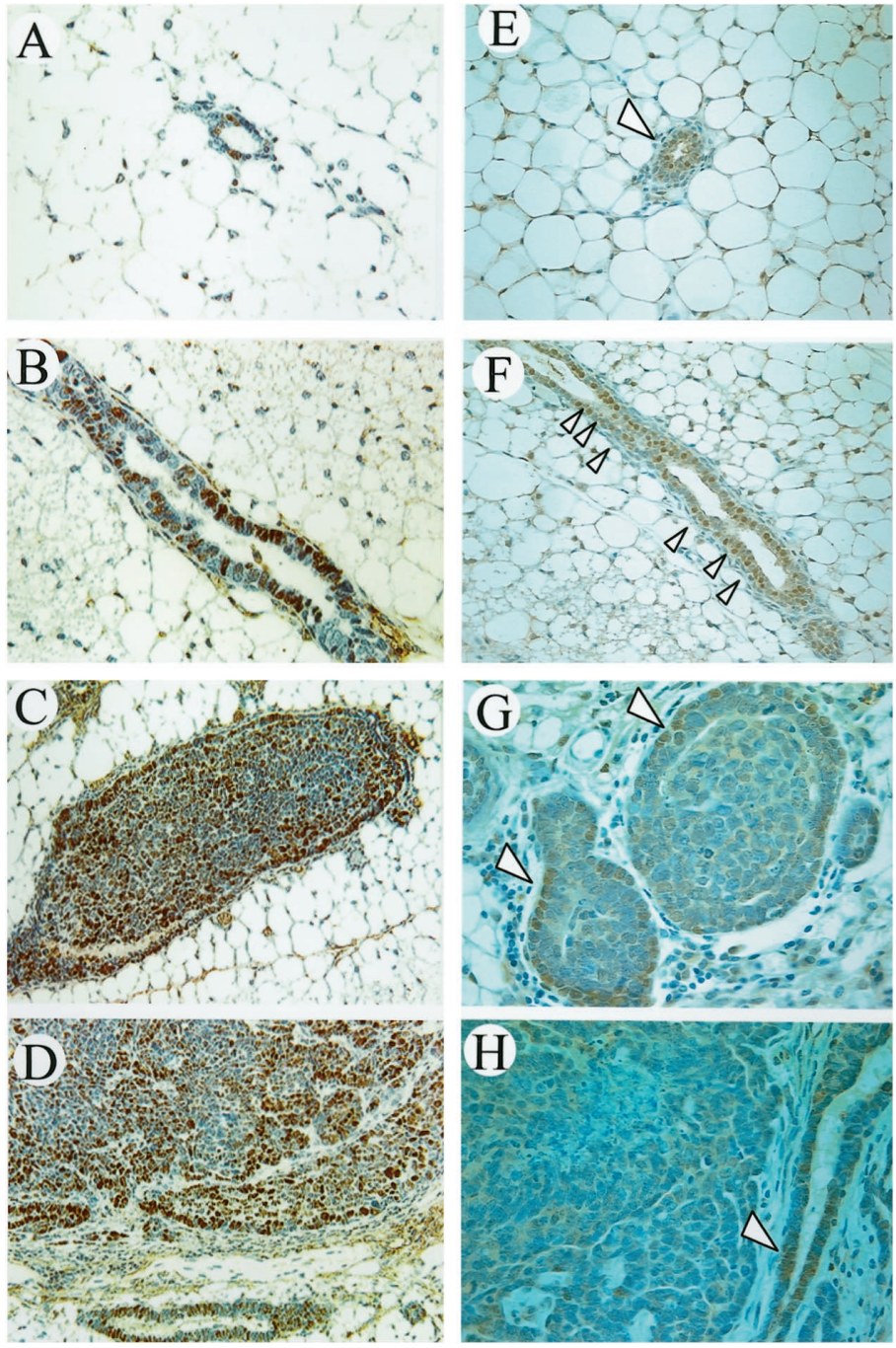

Fig. 2. Expression of T_{AG} and ERα by immunohistochemistry during mammary tumor development in C3(1)/T_{AG} transgenic mice. T_{AG} (*A–D*) and ERα (*E–H*) are shown. *A* and *E*, expression of T_{AG} and ERα was detectable at 3 weeks of age in normal-looking epithelium of intact female transgenic mice. *B* and *F*, at 8 weeks of age, low-grade MIN lesions were observed, and the number of T_{AG}-immunoreactive mammary epithelial cells increased. ERα was clearly detectable (*F*, *open arrows*). *C* and *G*, at 14 weeks of age, high-grade MIN are observed in all animals in all groups, whereas very few invasive carcinomas are observed at this age. Although nuclear T_{AG} expression was observed in most cells in MIN lesions, ERα is only detected in the basal layer of the mammary duct (*G*, *open arrow*). *D* and *H*, at 20 weeks of age, invasive carcinomas continued to express very high levels of T_{AG} throughout the tumor (*D*), but ERα expression was low or undetectable. The adjacent duct expressed ERα (*H*, *open arrow*).

glands of intact FVB/NCr female mice were analyzed and used as normal controls. A 1.8-kb *Eco*RI fragment of ERα fragment from pSG5-MOR was labeled with [α-^{32}P]dCTP by random oligonucleotide-primed synthesis. Poly(A) RNA was isolated using the MicroPoly(A) Pure kit (Ambion, Austin, TX) according to the manufacturer's protocol, and 5 μg of mRNA were fractionated on a 1.0% formaldehyde agarose gel, transferred onto a nylon membrane, and fixed by UV cross-linking. Membranes were hybridized with a ^{32}P-labeled probe and washed using standard protocols. The membranes were then exposed to X-ray film at -70°C for varying periods of time.

For RT-PCR, 2 μg of total RNA were incubated with DNAse I, then incubated with reverse transcriptase using oligo $(dT)_{12-18}$ primer and Super Script II, and subsequently incubated with RNaseH (Life Technologies, Inc.) according to the manufacturer's protocol. cDNA was amplified using a thermal cycler (PTC-100; M. J. Research, Inc., Watertown, MA). The conditions and the sequences for each primer set were as follows: T_{AG}: 32 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min; the 5′ primer corresponds to SV40 T_{AG} nucleotides 4198–4219 (5′-gcagacactctatgcctgtgtg-3′) and the 3′ primer corresponds to nucleotides 5040–5018 (5′-catcctgataaaggaggagatg-3′); ERβ: 35 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min; the 5′ primer corresponds to mouse ERβ (U81451) nucleotides 39-60 (5′-aactacagtgttcccag-cagca-3′) and the 3′ primer corresponds to nucleotides 311-290 (5′-atccctctt-ggcgcttggacta-3′); TGF-α: 32 cycles at 94°C for 1 min, 57°C for 2 min, and 72°C for 3 min; the 5′ primer corresponds to mouse TGF-α (U65016) nucleotides 117-137 (5′-cctgctagcgctgggtatcct-3′) and the 3′ primer corresponds to nucleotides 343-323 (5′-ctgcatgctcacagcgaacac-3′); EGFR: 32 cycles at 94°C for 1 min, 57°C for 2 min, and 72°C for 3 min; the 5′ primer corresponds to mouse EGFR (X59698) nucleotides 256-276 (5′-ggcacaagtaacaggctcacc-3′) and the 3′ primer corresponds to nucleotides 532-512 (5′-agttggacaggatggcta-agg-3′); her-2/neu: 32 cycles at 94°C for 1 min, 57°C for 2 min, and 72°C for 3 min; the 5′ primer corresponds to rat her-2/neu (X03362) nucleotides 1487-1504 (5′-cggaacccacatcaggcc-3′) and the 3′ primer corresponds to nucleotides 2179-2159 (5′-tttcctgcagcagcctacgc-3′); c-*myc*: 32 cycles at 94°C for

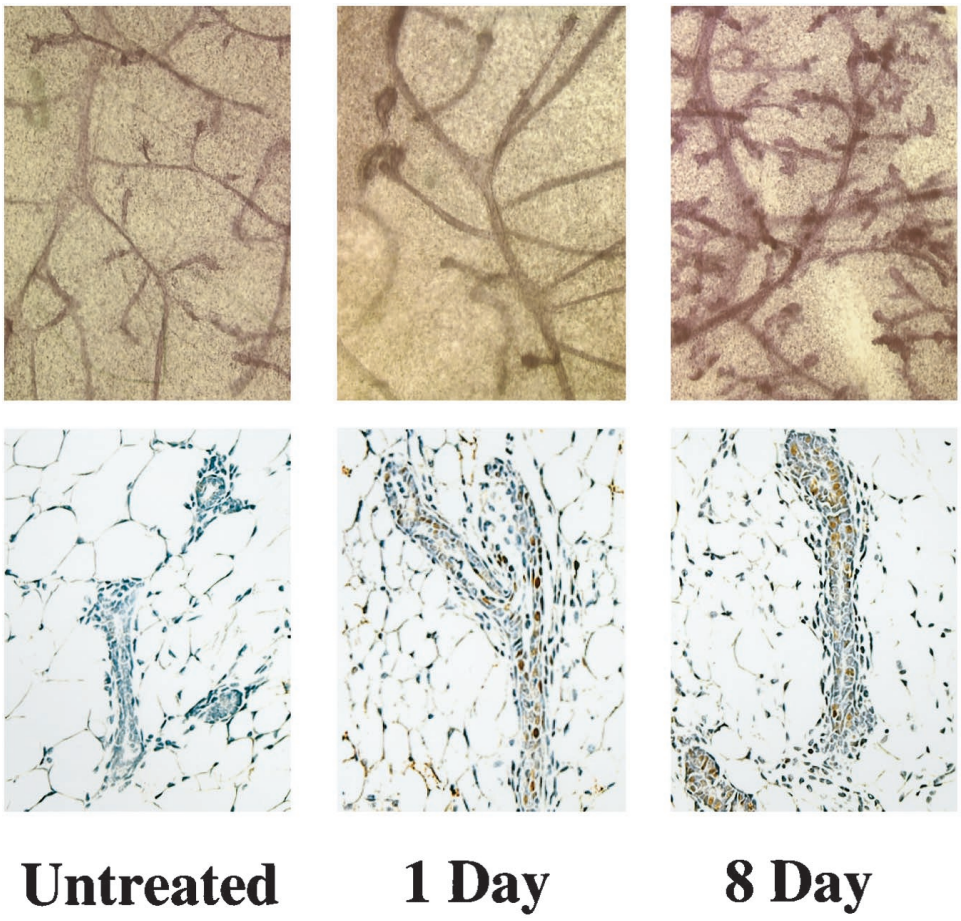

Fig. 3. Effect of E_2 on normal mammary gland epithelial cellularity and proliferation. Ten-week-old FVB/N mice were given supplemental E_2 as described in "Materials and Methods" for either 1 or 8 days. *Upper panels*, increased cellularity at the TDLUs was noted after 8 days of E_2 treatment, although alveolar differentiation was not apparent. *Lower panels*, immunohistochemical staining demonstrates an increasing number of cells expressing nuclear proliferating cell nuclear antigen after 1 and 8 days of E_2 treatment.

1 min, 57°C for 2 min, and 72°C for 3 min; the 5′ primer corresponds to mouse c-*myc* (X01023) nucleotides 1301-1324 (5′-gggccagccctgagcccctagtgc-3′) and the 3′ primer corresponds to mouse nucleotides 1456-1433 (5′-atggagatgagcccgactccgacc-3′); and G3PDH: 30 cycles at 94°C for 45 s, 60°C for 45 sec, and 72°C for 2 min; the 5′ primer used corresponds to mouse G3PDH (M32599) nucleotides 51-76 (5′-tgaaagtcggtgtgaacggatttggc-3′) and the 3′ primer to nucleotides 1033-1010 (5′-catgtaggccatgaggtccaccac-3′).

Statistical Analysis. The two-sided Student's *t* test was used to determine whether significant differences existed between the mean values of the groups analyzed. The log-rank test was used to evaluate the survival curves.

RESULTS

The C3(1) 5′ Flanking Sequence Is Unresponsive to E_2 *in Vitro* and *in Vivo*. To determine whether estrogen influences the natural history of tumor progression in C3(1)/T_{AG} transgenic mice independent of transgene expression, we examined what effect the estrogen signaling pathway might have on the transcriptional activity of the C3(1) regulatory regions. To examine the responsiveness of the C3(1) 5′ flanking region to stimulation by E_2 *in vitro*, we used two approaches. The M6 cell line, established from a C3(1)/T_{AG} mammary adenocarcinoma,[4] contains integrated copies of the C3(1)/T_{AG} transgene and expresses T_{AG}. Studies were performed to determine whether the transgene could be stimulated to produce increased amounts of T_{AG} by the addition of E_2. The addition of up to 20 nM E_2 to the media did not increase the level of T_{AG} protein produced by M6 cells, as determined by Western blot analysis (Fig. 1*A*, *Lanes 1–5*). However, M6 cells express low levels of endogenous ERα (Fig. 1, *Lane 1*), which is down-regulated by the addition of E_2 in a dose-dependent manner (Fig. 1*A*, *Lanes 1–5*), demonstrating that the ERα signaling pathway is still operative in these cells.

Because these cells expressed relatively low levels of ERα, which could limit the response of the transgene to stimulation by E_2, we performed experiments where high levels of murine ERα were expressed after transfection of the plasmid pCMVmERα. Despite the overexpression of ERα in M6 cells under these conditions, the addition of up to 20 nM E_2 did not lead to a change in T_{AG} levels (Fig. 1*A*, *Lanes 6–10*). These results demonstrate that although estrogen signaling is active in the M6 cells as evidenced by reduced ERα expression in response to E_2 (Fig. 1, *Lanes 1–5*), transcriptional activity of the C3(1) 5′ flanking sequence is not influenced by ER signaling in these cells (Fig. 1, *Lanes 1–10*).

The effect of E_2 stimulation of the transgene was also studied *in vivo*. Because E_2 stimulation of cell proliferation, either by direct stimulation of transgene expression or through other physiological mechanisms, would lead to an expansion of mammary gland lesions expressing T_{AG} protein, absolute levels of T_{AG} would not necessarily reflect an increase in transcriptional activity of the C3(1) 5′ flanking sequence. Therefore, to examine how transcriptionally responsive the C3(1)/T_{AG} transgene was to E_2 *in vivo*, it was necessary to perform *trans*-activation experiments over a relatively short time frame before significant proliferation could occur. Ten-week-old intact transgenic females were ovariectomized 1 week prior to being given a single intraperitoneal injection of 3 mg/kg body weight of E_2. T_{AG} expression in the mammary gland did not change after ovariectomy or within 24 h after injection of E_2 (Fig. 1*B*, *Lanes 1–8*). ERα expression increased with ovariectomy alone but significantly decreased in ovariectomized mice given E_2 supplementation (Fig. 1*B*, *Lanes 2, 6–8*). These results demonstrate that although the addition of estrogen reduces ERα expression in the mammary tissue as expected, C3(1) is not transactivated by E_2 *in vivo*. Thus, phenotypic effects observed in

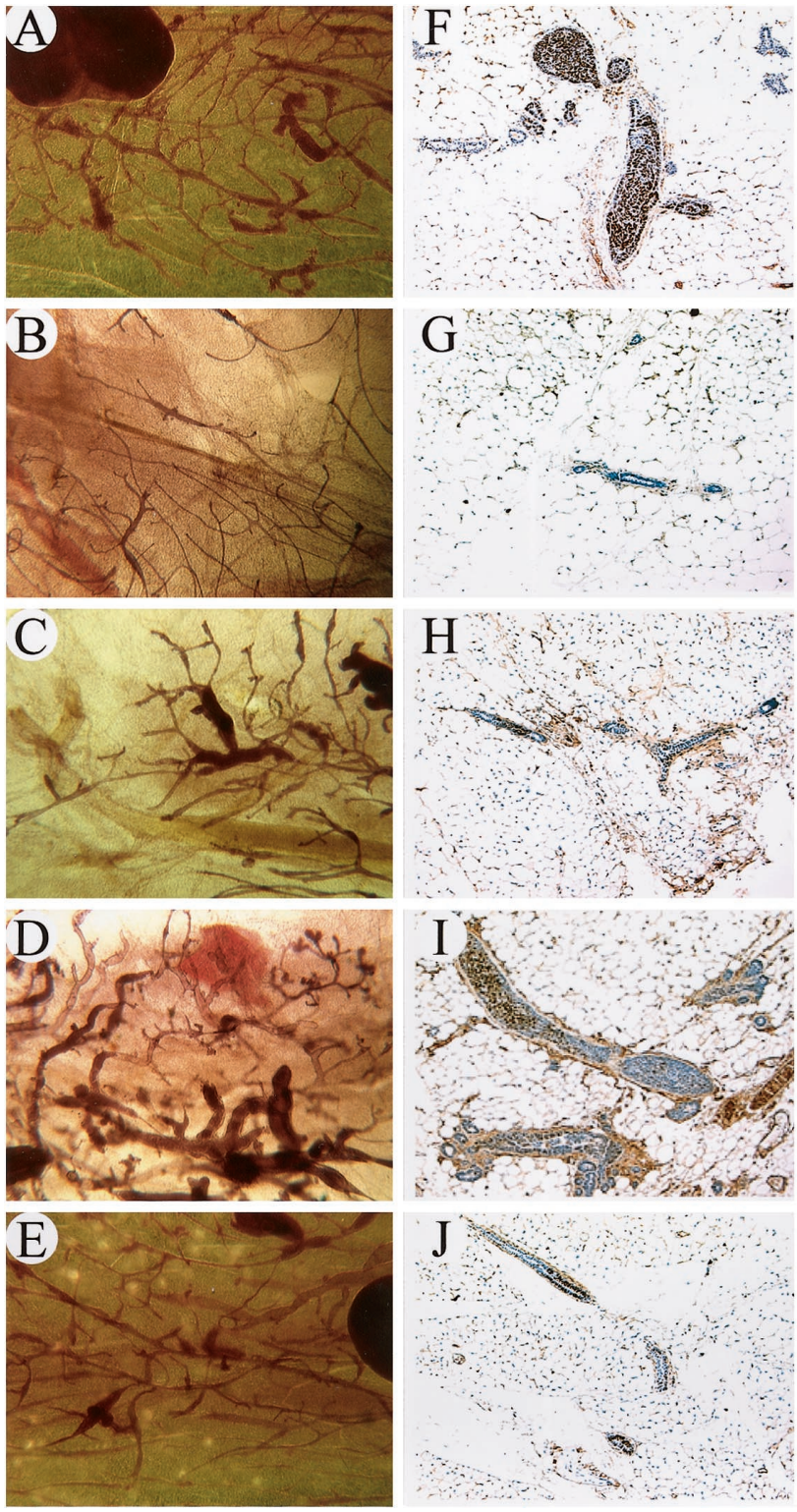

Fig. 4. Effect of hormone manipulations on mammary tumor development. Whole-mount preparations (*A–E*) and T_{AG} immunohistochemistry (*F–J*) are shown. *A* and *F*, full development of mammary ductal tree at 17 weeks of age with TDLU formation in intact C3(1)/T_{AG} transgenic female mice. Multiple ductal and TDLU lesions are observed. *B* and *G*, reduced branching with very few TLDUs and alveolar cells in OVX3W mice. *C* and *H*, OVX8W had normal branching but reduced alveolar cell formation with development of ductal lesions. *D* and *I*, E_2 supplementation restored branching and TLDU formation after ovariectomy at 8 weeks of age and induced significantly more ductal lesions. *E* and *J*, TAM treatment in an intact mouse inhibited TLDU formation, but lesions within ducts still formed. ×100.

C3(1)/T_{AG} mice given estradiol supplementation do not result from increased transgene expression.

Alterations in the Natural History and Histopathology of Mammary Tumor Formation in C3(1)/T_{AG} Mice by Estrogen Signaling. T_{AG} was not detectable by immunohistochemical analysis at 1, 8, or 14 days after birth (data not shown) but was detectable at 3 weeks of age in normal-looking epithelium of intact C3(1)/T_{AG} transgenic female mice (Fig. 2*A*). The number of T_{AG}-positive mammary epithelial cells increases with age and is associated with multifocal low-grade MIN lesions (12) in the ducts and TDLUs of the mammary gland beginning at about 8 weeks of age (Fig. 2*B*). These lesions progress to advanced MIN by 14 weeks of age (Fig. 2*C*). There is a progressive increase in the number of MIN lesions that begin to develop into invasive mammary adenocarcinomas at about 17 weeks of age. These tumors continue to express high levels of T_{AG} (Fig. 2*D*). Gross tumors were generally observed after 16 weeks of age, with mice dying by 7 months of age because of tumor burden. The mammary carcinomas are morphologically designated as mixed solid

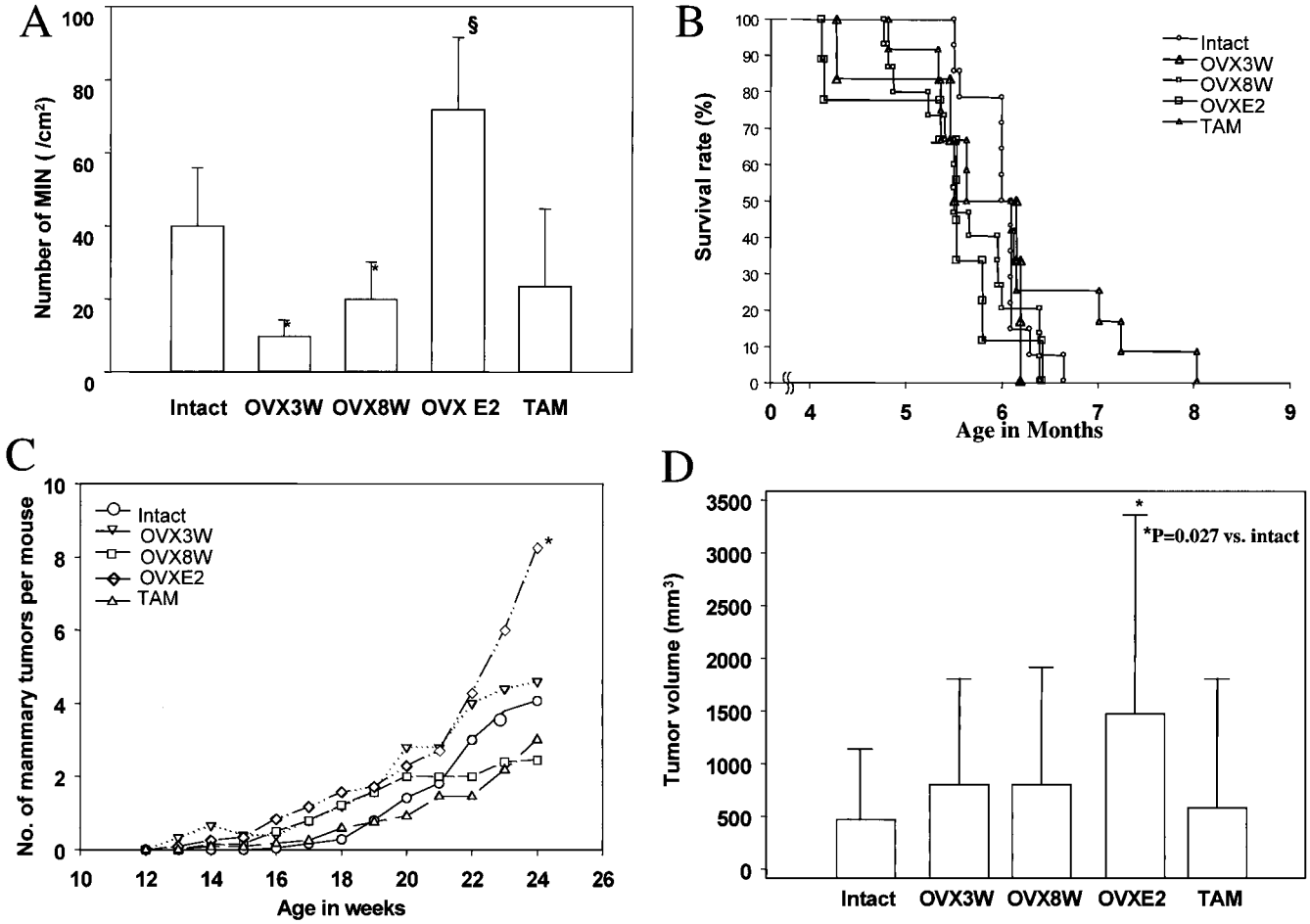

Fig. 5. Histopathological analysis of hormone-manipulated C3(1)/T_{AG} transgenic mice. *A*, number of microscopic MIN lesions at 14 weeks of age. $OVXE_2$ mice had a significantly higher number of MIN lesions compared with all other groups (§, $P = 0.0026$ *versus* intact; $P = 0.00003$ *versus* OVX 3W; $P = 0.00013$ *versus* OVX8W; and $P = 0.0037$ *versus* TAM). OVX3W and OVX8W showed significantly fewer MIN lesions compared with intact mice at this stage (*, $P = 0.00031$ and $P = 0.0077$, respectively). *B*, survival rates. *C*, number of palpable tumors by age. *D*, palpable tumor volume at 20 weeks of age. There was no difference in survival rate, number of palpable tumors, or tumor volumes between intact. OVX3W, OVX8W, and TAM groups. $OVXE_2$ had a significantly shorter survival period ($P = 0.015$), increased tumor multiplicity ($P = 0.016$), and tumor volumes ($P = 0.0266$) compared with intact mice. *Bars*, SD.

and glandular carcinomas with necrosis and fibrosis (12) and appear to resemble what has been called poorly to moderately differentiated human invasive ductal adenocarcinomas.

To investigate how changes in estrogen exposure might alter mammary cancer progression in this model, animals were treated to reduce or stimulate effective estrogen levels. We confirmed that these manipulations of estrogen levels had biological effects by measuring uterine weight. OVX3W, OVX8W, or TAM therapy reduced uterine weight as measured at 11 weeks of age, whereas administration of E_2 increased uterine weight (intact, 48.8 ± 22.6 mg; OVX3W, 15.0 ± 2.8 mg; OVX8W, 18.5 ± 5.5 mg; TAM, 20.2 ± 5.6 mg; OVX E_2, 117.7 ± 9.3 mg).

The administration of supplemental E_2 to normal intact FVB/N females at 10 weeks of age resulted in proliferative effects in the TDLUs, leading to increased proliferation and cellularity (Fig. 3) without expansive lobuloalveaolar formation as is seen in pregnancy. Proliferating cell nuclear antigen positivity by immunohistochemical staining rose from 3.7% of mammary epithelial cells in untreated mice, to 9.0 and 21.4% of cells in mice treated with E_2 for 1 or 8 days, respectively. This result demonstrates that estrogen can provide a proliferative signal to the mammary epithelium and increase the number of target cells, where the C3(1)/T_{AG} transgene is transcribed.

Whole-mount preparations and H&E sections of intact C3(1)/T_{AG} female mammary glands at 17 weeks of age showed complete development of mammary ducts and TDLU formation with MIN and invasive carcinomas arising in both the ducts and TDLUs (Fig. 4, *A* and *F*). Ovariectomy before puberty (3 weeks of age) resulted in a dramatic decrease in mammary duct side-branching and alveolar bud formation (Fig. 4, *B* and *G*) with a reduction in the number of MIN lesions compared with intact animals (Fig. 5*A*). Ovariectomy after puberty (8 weeks of age) resulted in a more modest reduction in duct and TDLU formation and MIN formation (Fig. 4, *C* and *H*). At 14 weeks of age, the number of MIN lesions in the OVX3W and OVX8W groups were significantly lower than that of the intact group ($P = 0.00031$ and $P = 0.0077$, respectively; Fig. 5*D*). There was also a trend toward a lower number of MIN lesions in OVX3W animals compared with that of OVX8W, although this was not statistically significant ($P = 0.054$).

Mice ovariectomized at 8 weeks of age and given E_2 supplementation demonstrated more ductal branching and TDLU formation than intact animals (Fig. 4*D*), which was also associated with a higher number of MIN lesions in the E_2-supplemented group (Fig. 4*I*) compared with the other groups (Fig. 5*D*; $P = 0.0026$ *versus* intact; $P = 0.00003$ *versus* OVX 3W; $P = 0.00013$ *versus* OVX8W; and $P = 0.0037$ *versus* TAM). Tamoxifen treatment given to intact females at 8 weeks of age moderately inhibited TDLU formation (Fig. 4*E*) and tended to reduce MIN formation (Fig. 4*J*) compared with

intact animals, although this was not statistically significant ($P = 0.075$; Fig. 5*A*).

Ovariectomized female mice given E_2 supplementation died significantly earlier than intact mice ($P = 0.0157$; Fig. 5*B*) because of accelerated tumor formation associated with increased tumor multiplicity ($P = 0.016$; Fig. 5*C*) and tumor volumes (Fig. 5*D*). The OVX E_2 group had significantly larger tumors than did the intact group ($P = 0.0266$) at 20 weeks of age, but there was no significant difference among the other groups (Fig. 5*D*). Although both the 3-week-old and 8-week old ovariectomized groups that did not receive E_2 had a reduced number of MIN lesions at 14 weeks of age compared with controls, the actual number of palpable tumors was not significantly different from that observed in the intact group (Fig. 5*C*). By 20 weeks of age, 15 of 16 OVX3W mice had developed palpable mammary tumors. This suggests that by 3 weeks of age, the number of target cells for transgene expression leading to the development of gross tumor formation at 20 weeks of age was not influenced by ovariectomy. However, estrogen supplementation promoted a large increase in the number of MIN lesions as well as an acceleration of tumor development.

E_2 supplementation to ovariectomized C3(1)/T_{AG} mice also resulted in more aggressive lesion formation as assessed by histopathology. E_2 given to mice ovariectomized at 8 weeks of age induced significantly more high-grade MIN lesions and invasive carcinomas than untreated ovariectomized mice (data not shown). Small, early MIN lesions showed a higher grade of cellular atypia with a loose pattern of cell-cell interaction compared with early lesions arising in intact mice. In a subgroup (17%) of the mice supplemented with E_2, ectopic chondrocyte and bone formation was noted. This incidence is much higher than the occasional ectopic chondrocyte and bone formation observed in mammary glands from control C3(1)/T_{AG} female mice (<1%; Ref. 6).

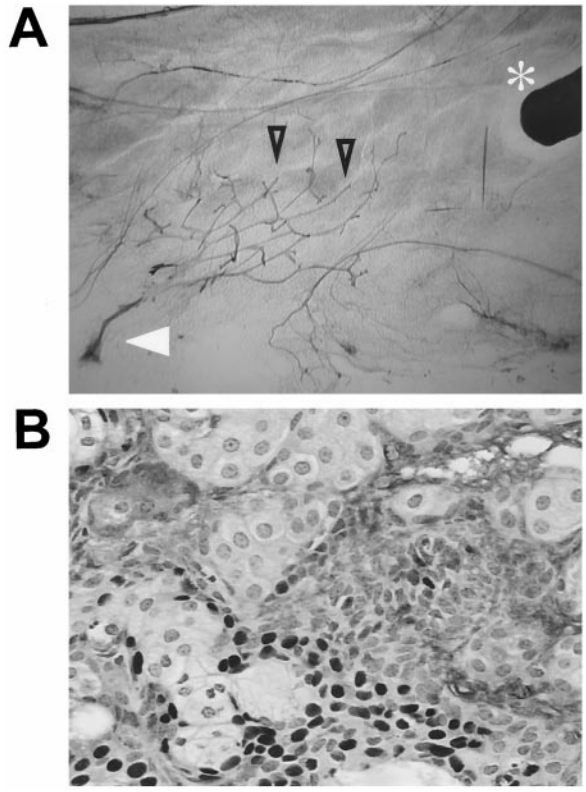

Fig. 6. Mammary and salivary gland lesions in C3(1)/T_{AG}-ER$\alpha^{-/-}$ (ERKO) female mice. *A*, mammary gland whole-mount preparation of C3(1)/T_{AG}-ER$\alpha^{-/-}$ female demonstrates only primitive mammary ducts below the nipples. *B*, T_{AG} was highly expressed in salivary gland tumors from C3(1)/T_{AG}-ER$\alpha^{-/-}$ female mice. *Open arrowheads*, primitive ducts; *closed arrowhead*, nipple area; *asterisk*, lymph node region.

Effect of the Loss of ERα Alleles on Mammary Tumor Progression. Because the prepubertal removal of estrogen by ovariectomy at 3 weeks of age did not significantly reduce palpable mammary tumor formation (Fig. 5*C*), experiments were performed to determine whether mammary tumor development induced by the C3(1)/T_{AG} transgene could occur in rudimentary mammary ductal *anlage* lacking functional ERα. Mice genetically engineered to lack functional ERα (αERKO) exhibit mammary gland hypoplasia with development arrested prior to ductal branching (13, 14). To determine whether the C3(1)/T_{AG} transgene would be expressed and lead to tumor formation in this mammary epithelium where development was arrested at an early stage prior to ductal branching. C3(1)/T_{AG} mice were generated in the ERKO background. Previous studies using MMTV-int-1-αERKO mice have demonstrated that mammary tumors develop in the absence of ERα (15).

C3(1)/T_{AG}-ER$\alpha^{+/+}$ females in the FVB/N-C57BL6 mixed background lived significantly longer than C3(1)/T_{AG}-ER$\alpha^{+/+}$ females in the FVB/N background, suggesting that there are important strain differences that modulate tumor development. Whole-mount preparations of the mammary glands of C3(1)/T_{AG}-ER$\alpha^{-/-}$ (αERKO) female mice demonstrated that a primitive mammary ductal system developed with the formation of only secondary branches near the nipples (Fig. 6*A*). T_{AG} expression was not detected by immunohistochemistry or by RT-PCR in the rudimentary mammary ductal epithelium of these mice up to 9 months of age (data not shown). No proliferative lesions were observed in these rudimentary mammary structures by histopathological analysis at any time during the 10-month period of study. These results indicate that the C3(1)/T_{AG} transgene is not expressed in the very rudimentary mammary ductal structures prior to the onset of ductal branching. The transgene is expressed when the mammary epithelial cells progress to a more differentiated state at 3 weeks of age. ERα is required for this early differentiation and outgrowth of the ductal cells. C3(1)/T_{AG} mice that were heterozygous or wild-type for ERα developed normal mammary glands, and mice from both genotypes exhibited a similar incidence of mammary gland tumor formation (56.7% tumor incidence for C3(1)/T_{AG}-ER$\alpha^{+/-}$ and 67.8% tumor incidence for C3(1)/T_{AG}-ER$\alpha^{+/+}$ mice).

Although no mammary tumors were observed in C3(1)/T_{AG}-αERKO females, tumors developed in other organs of C3(1)/T_{AG}-αERKO mice similar to those observed in C3(1)/T_{AG} ER$\alpha^{+/+}$ in the FVB/N background. C3(1)/T_{AG}-αERKO female mice developed foot pad tumors (23.5%) and salivary gland tumors (5.9%), which expressed high levels of T_{AG} (Fig. 6*B*). This observation further suggests that the ER signaling pathway is not required for expression of the C3(1)/T_{AG} transgene. However, unlike C3(1)/T_{AG} mice in the FVB/N background, carcinomas of the nasal structures were more frequently observed in the hybrid FVB/N $\times$ C57BL6 background (<1% *versus* 64.7%, respectively). Animals were sacrificed because of the morbidity associated with these nonmammary tumors.

There was no significant difference in survival rates among C3(1)/T_{AG} transgenic mice with either the *ER*$\alpha^{+/+}$, *ER*$\alpha^{+/-}$, or *ER*$\alpha^{-/-}$ genotypes. All C3(1)/T_{AG} mice died before 10 months of age, regardless of the ERα genotype. Death was either because of the development of mammary tumors in mice carrying the *ER*$\alpha^{+/+}$ or *ER*$\alpha^{+/-}$ genotypes or because of salivary and nasal tumors in C3(1)/T_{AG} mice carrying the *ER*$\alpha^{-/-}$.

ERα Expression Is Reduced during Mammary Tumor Progression. ERα expression was observed only in mammary epithelial cells with normal morphology (Fig. 2*E*) and low-grade MIN lesions in C3(1)/T_{AG} transgenic mice (Fig. 2*F*). There was a dramatic decrease

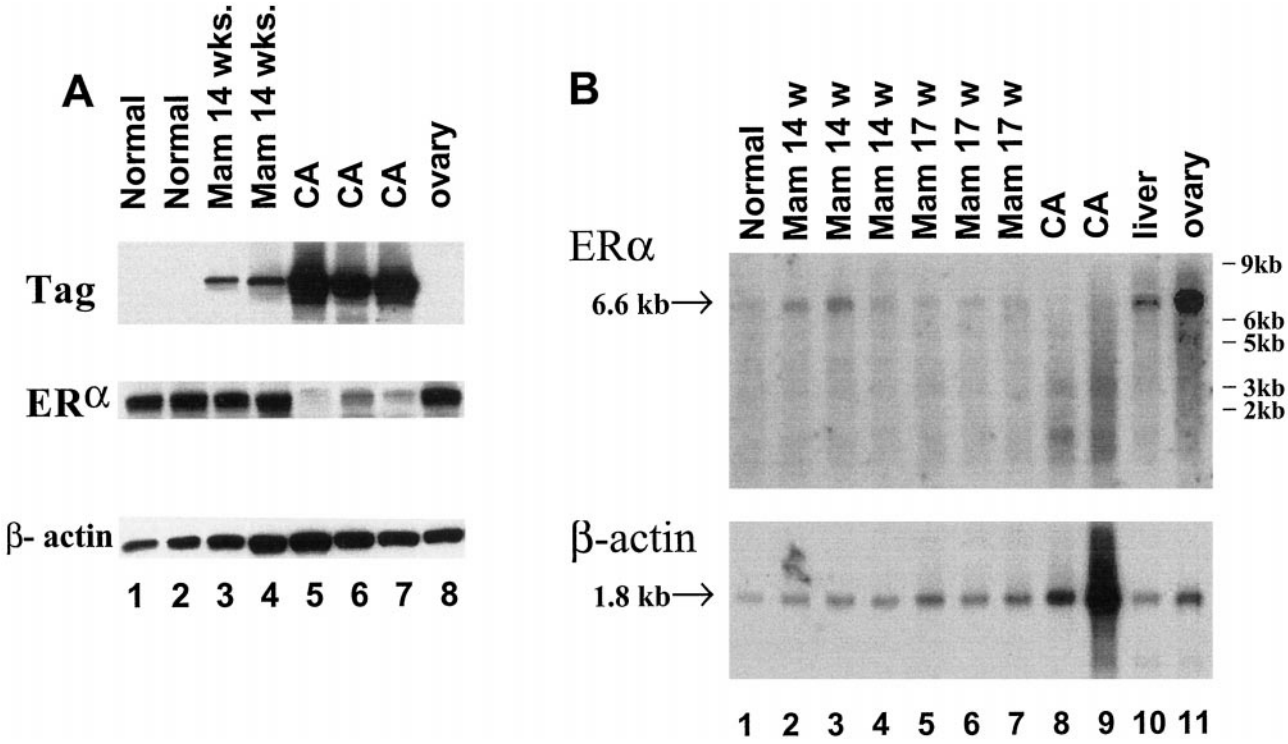

Fig. 7. Analysis of T_{AG} and ERα during mammary tumor development. *A*, expression of T_{AG} increases during mammary tumor development, whereas ERα expression was significantly decreased in tumors. *Lanes 1* and *2*, normal FVB/N mammary gland; *Lanes 3* and *4*, C3(1)/T_{AG} mammary gland with MIN at 14 weeks of age (*Mam 14 wks*.); *Lanes 5–7*, C3(1)/T_{AG} mammary tumors (*CA*); *Lane 8*, C3(1)/T_{AG} ovary. *B*, Northern blotting for ERα and β-actin. Five μg of poly(A) RNA was electrophoresed through a 1% agarose gel and hybridized with a ^{32}P-labeled probe for ERα as described in "Materials and Methods." *Lane 1*, mammary gland from a normal FVB/N female; *Lanes 2–4*, C3(1)/T_{AG} mammary gland with MIN at 14 weeks of age (*Mam 14 w*); *Lanes 5–7*, C3(1)/T_{AG} mammary gland with invasive carcinomas (*CA*) at 17 weeks of age; *Lanes 8–9*, C3(1)/T_{AG} mammary tumors; *Lane 10*, liver; *Lane 11*, ovary. mRNA (6.6 kb) of ERα was significantly decreased in mammary tumors.

in ERα expression in high-grade MIN lesions, with some cells demonstrating weak nuclear staining, although normal neighboring luminal cells stained strongly positively for ERα (Fig. 2*G*). ERα expression in invasive carcinomas was low to undetectable (Fig. 2*H*). A similar reduction in ERα expression was observed during mammary tumor progression, as assessed by Northern and Western blot analyses (Fig. 7). Expression of T_{AG} protein increased during mammary tumor progression, as determined by Western blot analysis (Fig. 7*A*), whereas ERα expression was dramatically decreased in tumors compared with normal FVB/N mammary glands or preneoplastic lesions. Northern blotting also demonstrated that steady-state levels of a 6.6-kb ERα transcript were significantly lower in tumors compared with normal tissue and preneoplastic lesions, suggesting that the down-regulation of ERα occurs at the RNA level (Fig. 7*B*). Because normal mammary tissue and tissues with MIN lesions contain a much smaller percentage of epithelial cells compared with the carcinomas, the relative reduction in ERα expression in the tumor cells is presumably even greater than that depicted by these results.

The expression of ERα was inversely related to estrogen exposure. ERα was elevated in tumors that developed with reduced exposure to estrogen (OVX) compared with tumors from unmanipulated transgenic animals (Figure 1*B, Lane 1* compared with *Lane 2*). E_2 supplementation to OVX mice tended to suppress expression of ERα (Fig. 1*B, Lane 5* compared with *Lane 2*). ERα expression tended to be increased in the TAM group compared with that of intact animals (data not shown). T_{AG} expression in mammary tumors from intact and OVX3W mice was similar, further demonstrating that transgene expression was not dependent upon estrogen signaling (Fig. 1*C*).

ERβ expression as determined by RT-PCR was barely detectable in normal mammary tissue but appeared to increase during mammary tumor progression (Fig. 8). ERβ expression was not significantly altered in tumors arising from the hormone-manipulated groups.

Alterations in the Expression of Proto-Oncogenes and Hormone Receptors during Mammary Tumor Progression. Altered expression of several oncogenes has been shown to be associated with tumor progression and clinical outcome in human breast cancer. To determine whether similar changes in the expression of several relevant genes might occur during mammary tumor progression in this transgenic model and whether alterations in estrogen exposure might modify the expression of these genes, RT-PCR analyses were performed. Overexpression of several proto-oncogenes frequently associated with human mammary cancers was also found in the C3(1)/T_{AG} transgenic mammary tumors. These include c-myc, her-2/neu, TGF-α, and EGFR. All the reactions were semiquantitatively confirmed by serial dilution of template cDNA samples using the same number of cycles. Expression of TGF-α and EGFR appear to increase in early lesions, whereas expression of c-myc and neu appears to increase at the invasive carcinoma stage (Fig. 8). There was no difference in the expression of these genes in tumors derived from mice in which estrogen levels were increased or decreased compared with tumors derived from intact mice (data not shown). However, we do not know whether expression of these genes might have been altered by changes in estrogen levels at earlier stages of tumor progression.

DISCUSSION

It is primarily through epidemiological evidence that estrogen has been implicated in stimulating mammary oncogenesis (3). It has not been possible to study the role of estrogen in the promotion of mammary cancer in most transgenic models because they generally use the hormone-responsive MMTV, whey acidic protein, and β-lactoglobulin promoters. Although MMTV may be active in the virgin mammary gland, phenotypic expression often requires the use of hormones or pregnancy to substantially increase MMTV transcrip-

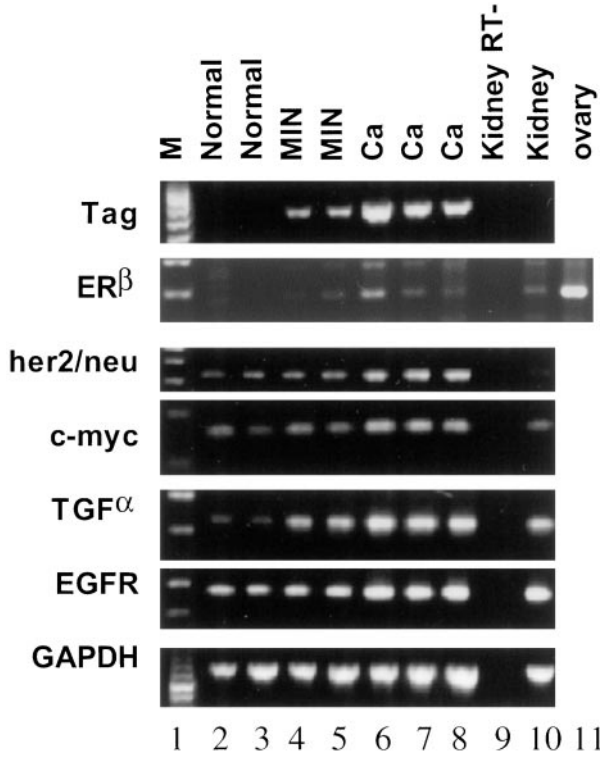

Fig. 8. Alterations proto-oncogene and hormone receptor expression. RT-PCR expression analyses for T_{AG} and *ERβ, her2/neu,* c-*myc, TGFα,* EGFR, and G3PDH (*GAPDH*) during tumor progression in unmanipulated mice. *Lane 1,* marker; *Lanes 2* and *3,* normal FVB/N mammary gland; *Lanes 4* and *5,* C3(1)/T_{AG} mammary gland with MIN at 14 weeks of age; *Lanes 6–8,* C3(1)/T_{AG} mammary tumors (*Ca*); *Lane 9,* kidney of FVB/N female without reverse transcriptase (*Kidney RT−*); *Lane 10,* with RT; *Lane 11,* normal ovary.

tional activity (16). Whey acidic protein and β-lactoglobulin promoters have been derived from milk-specific genes and, therefore, are also most highly expressed during pregnancy and lactation (7). In addition, these promoters primarily target the alveolar epithelium and induce phenotypes in alveolar cells.

The C3(1)/T_{AG} transgene, however, is active in ductal epithelial cells, terminal end buds, and the TLDUs in virgin mammary glands. In this study, we have demonstrated both *in vitro* and *in vivo* that the C3(1) 5′ flanking region is not *trans*-activated by estrogen. This is consistent with a previous study where the endogenous expression of C3(1) in the rat was shown to be stimulated by androgen but not by estrogen (17). Earlier studies of the *C3(1)* gene demonstrated that the major hormone response element is contained within the first intron (18, 19), which is not contained in the C3(1)/T_{AG} transgene used in this model. Our results demonstrate that the C3(1) 5′ flanking region is not responsive to estrogen stimulation as evidenced by the following: (*a*) T_{AG} expression is maintained in tumors and tumor cell lines despite the loss of ERα expression; (*b*) estrogen does not stimulate transgene expression in transgenic mammary glands or transgenic mammary cells lines, even with the cotransfection of ERα. ER signaling is maintained, however, as evidenced by the down-regulation of endogenous ERα expression in response to E_2 and the up-regulation ERα in the presence of anti-estrogen therapy; (*c*) C3(1)/T_{AG} expression and tumor formation occur in several different organs in genetically modified mice completely lacking functional ERα; and (*d*) prepubertal ovariectomy resulted in similar rates of gross tumor formation as that observed in intact animals.

Expression of the C3(1)/T_{AG} transgene occurs only in mammary target cells that have reached the ductal outgrowth stage. Mice containing the transgene but lacking ERα develop only vestigial ducts present at the nipples without ductal outgrowth (20). The C3(1)/T_{AG} transgene is not transcribed in these rudimentary mammary anlagen, and tumors do not develop in contrast to MMTV-int-1/ERKO mice where tumors arise (15). However, in mice containing ERα where ductal outgrowth occurs, the C3(1)/T_{AG} transgene is expressed, and tumors form when mice are ovariectomized as early as 3 weeks of age. Thus, full maturation of the mammary epithelium is not necessary for tumor formation.

Although E_2 did not increase expression of the transgene, E_2 induced significantly more MIN lesions, invasive carcinomas, larger tumors, and reduced survival. These effects of estrogen may be attributable to an increase in the number of target cells that express the transgene and/or by promotion of tumorigenesis through other stimulatory mechanisms. It is also possible that the stimulation of tumorigenesis by estrogen is mediated through paracrine mechanisms involving the stroma. We have demonstrated that E_2 causes proliferative changes in the mammary epithelium, in particular by increasing cell numbers in the TDLU. Because these cells are targets for C3(1)/T_{AG} expression, it seems likely that increased numbers of lesions induced by E_2 results at least in part from the expansion of the number of target cells. There is strong evidence that a similar phenomenon occurs in human breast cancer, where higher density of breast epithelium is associated with an increased risk of breast cancer (21). Hormone replacement therapy is associated with increased breast density (22, 23), which may be part of the mechanism of hormone replacement therapy in elevating the cumulative risk of developing breast cancer (2, 21).

The histological features of mammary lesions in the C3(1)/T_{AG} transgenic females share resemblance to human ductal carcinoma *in situ* and infiltrating ductal adenocarcinomas (8, 9). In addition to accelerated growth of mammary lesions, estrogen stimulation induced histological changes in the tumors. More severe nuclear atypism within MIN lesions was noted with E_2 treatment. From these results, it appears that E_2 accelerates the process of "malignant conversion" in this model. Although there was a trend toward a reduction in the number of MIN lesions and tumors arising in animals given TAM, this was not statistically significant. It is likely that the major population of target cells for transgene expression was already developed by 8 weeks of age when TAM treatment was begun, and that TAM, therefore, had little effect on lesion development compared with intact mice.

ERα is expressed in normal mammary ductal cells and in low-grade MIN lesions in which T_{AG} is expressed. However, in high-grade MIN and invasive carcinomas, the expression of ERα mRNA and protein is significantly reduced, as determined by Northern blotting, immunohistochemistry, and Western blotting. It, therefore, appears that reduced ERα expression occurs as part of the process of tumor progression and is not the result of a clonal outgrowth of epithelial cells that are initially ERα negative. To our knowledge, this is the first report demonstrating the loss of ERα expression during tumor progression in a transgenic model for mammary cancer. Human breast cancers often progress to an ERα-negative state that is generally associated with a more aggressive disease course and poorer clinical outcome because they rarely respond to hormone therapies (24).

The expression of the C3(1)/T_{AG} transgene does not increase in response to E_2 *in vitro* and *in vivo*, but E_2 does reduce endogenous ERα expression as expected. These data suggest that despite an intact estrogen signaling pathway, estrogen does not affect transcription of the C3(1)/T_{AG} transgene. The expression of T_{AG} did not appear to interfere with estrogen signaling because ERα expression diminished in response to exogenous E_2, despite the presence of T_{AG}.

Although it appears paradoxical that estrogen promotes tumorigenesis in this model whereas expression of ERα is reduced during tumor progression, these findings suggest that estrogen signaling may play

different roles in oncogenesis, depending upon tumor stage. In this model, it appears that estrogen can stimulate proliferation of the target tissue for transgene expression and may also result in the up-regulation of cellular factors that promote tumorigenesis following the initiation of oncogenesis by T_{AG}. Once this interaction occurs during the MIN stage, further progression to invasive carcinoma may no longer require the influence of estrogen and expression of ERα. Given the fact that ERα expression is consistently reduced during advanced stages of tumorigenesis, it is possible that there is selective pressure to down-regulate ERα expression to provide a growth advantage for invasive tumor development.

A second ER, ERβ, has been cloned from the rat prostate (25). ERβ mRNA is expressed in both normal and neoplastic human breast tissues (26, 27). In this study, ERβ expression was not detected in normal mammary glands but was detectable in transgenic mammary tumors. The role of ERβ in mammary tumorigenesis in this model will require further study.

We have demonstrated previously that mammary tumor progression in this model involves several genetic alterations including amplification and overexpression of Ki-ras (28),[6] loss of p53, Rb, and p21 function (9, 29)[7] and loss of protective apoptotic mechanisms (30). In this study, we have explored whether the expression of other genes relevant to human breast cancer are altered during mammary tumor progression in this model. The expression of her2/neu mRNA appeared to be slightly elevated in carcinomas, but expression in tumors was not affected by hormone manipulations. Both TGF-α and EGFR were up-regulated at the MIN stage, suggesting that an autocrine/paracrine mechanism involving this pathway might be an early event in this model of mammary cancer development. Expression of c-myc is up-regulated in the carcinomas but not in MIN lesions in this transgenic model, suggesting that c-myc overexpression may be a late event in mammary oncogenesis in this model. Hormone manipulations did not induce a change in the expression of c-myc in the mammary tumors, suggesting that the ERα pathway is not a major regulator of c-myc in this model.

In summary, mammary oncogenesis in the C3(1)/T_{AG} transgenic model appears to be promoted by estrogen through a mechanism that does not involve up-regulation of transgene expression by estrogen. The mechanism for this stimulatory effect of estrogen on tumorigenesis will be the focus of future studies. Further insights into how ERα expression is lost during tumor progression in this model may be highly relevant to understanding the transition in human breast cancer from an ERα-positive to an ERα-negative state.

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